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Reconstitution of photosynthetic charge accumulation and oxygen evolution in $CaCl_2$-treated PS II particles

I: Establishment of a high recovery of O_2 evolution and examination of the roles of the 17-, 23- and 34-kDa proteins, focusing on the effect of Cl^- on O_2 evolution

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The reconstitution of high O_2 evolution in $CaCl_2$-treated PS II particles was achieved by the simultaneous addition of the 17-, 23- and 34-kDa proteins and total thylakoid lipids in the presence of 25% glycerol and 15 mM sodium cholate. The activity of the reconstituted membranes recovered to 85% of that of the non-depleted original PS II particles at the optimal condition. By means of this reconstitution method, evidence for the cooperation of the three proteins in the recovery of O_2 evolution in the $CaCl_2$-treated PS II particles was found by changing the concentration of NaCl in the assay medium, and the relationship between the amount of manganese retained in the water-splitting complex and the O_2 evolving activity was examined by using the partially solubilized PS II particles with *n*-octyl-*β*-D-glucoside.

Photosystem II *O_2 evolution reconstitution* *$CaCl_2$-treatment* *17-kDa protein* *23-kDa protein* *34-kDa protein* *Manganese* n-*Octyl-β-D-glucoside*

1. INTRODUCTION

During the past two years, the constituents of the water-splitting complex in the thylakoid membranes have been studied mainly by means of the disintegration and reconstitution of the O_2 evolution and it has been found that the three proteins with molecular masses of 17-, 23- and 34-kDa, which locate at the inner surface of the thylakoid membranes, functionally associate with the water-splitting complex [1–11]. In early works however, there were some discrepancies regarding the requirements for the three proteins in O_2 evolution.

In [8] it was reported that the inhibition of the O_2 evolution in inside-out thylakoid was proportional to the amount of released 23-kDa protein, while authors in [3] showed the recovery of the O_2 evolution by simultaneous reinsertion of the 17- and 23-kDa proteins in cholate-treated thylakoids but not each protein alone. Authors in [10] claimed that for the PS II particles from which the 17- and 23-kDa proteins were completely depleted by NaCl washing, the activity remained at about 40% of the original level. Recently, authors in [11] succeeded in the complete removal of the three proteins from the PS II particles concomitant with complete inactivation of the O_2 evolution by washing with 1 M $CaCl_2$, leaving Mn unaffected. They also found

Abbreviations: Bistris, bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane; Mops, 3-(*N*-morpholino)-propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DPC, 1,5-diphenylcarbazide; DCIP, 2,6-dichlorophenolindophenol; PBQ, phenyl-*p*-benzoquinone; chl, chlorophyll; PS II, photosystem II; OG, *n*-octyl-*β*-D-glycoside

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that the O_2 evolution was recovered in the $CaCl_2$-treated PS II with rebinding of the 34-kDa protein alone, although the recovery was limited to 28% of the original level. The findings in [11,12] were consistent with the results in [10] but seemed to be in conflict with the results in [8] and [3]. More recently, authors in [13] found the requirement for the protein in the recovery of the O_2 evolution in cholate-treated thylakoid membranes to depend strongly on the concentration of Cl^- in the assay medium, and the authors in [14] also mentioned the requirement for the 23-kDa protein to be modified in the presence of Cl^- at as high a concentration as 250 mM. In both experiments, however, the 34-kDa protein was removed only in part from each original thylakoid membrane; consequently Cl^- dependency of the requirement for each protein in O_2 evolution was not examined separately. In order to examine the roles of the three proteins in the water-splitting process by means of reconstitution, it is necessary to establish the reconstitution of the attending function, with high recovery, in the totally depleted PS II particles regarding these proteins.

We report here a method to recover the O_2 evolution in $CaCl_2$-treated PS II particles at about 85% of the original level and apply it to obtain the relations among the three proteins and Cl^- for the O_2 evolution, and to examine the relation between the amount of manganese retained in the water-splitting complex and the O_2 evolving activity. In part II of this series, this reconstitution method will be used to show that the 17-, 23- and 34-kDa proteins are responsible for the $S_1 \rightarrow S_2$ transition of the water-splitting complex rather than other transitions by monitoring the EPR multiline signal in the reconstituted PS II particles prepared with different combinations of the proteins.

2. MATERIALS AND METHODS

PS II particles were prepared from spinach class 2 chloroplasts by a slight modification of the method in [6]. O_2 evolving activity of the particles prepared here was in the range of 320–450 μmol O_2/mg chl per h.

The mixture of 17-, 23-, 34-kDa proteins was extracted from the PS II particles as in [11]. These proteins were purified by using a Pharmacia FPLC system with columns of Mono Q (for 23- and 34-kDa proteins) and Mono S (for the 17-kDa protein). The effluent was 20 mM Bistris buffer (pH 7.0, 4°C) for the former, and 50 mM Mes buffer (pH 6.0 at 4°C) for the latter, with varying ionic strengths (NaCl, 0–0.5 M). Each protein sample was dialyzed against buffer solution A (0.2 M sucrose, 20 mM Mops, pH 7.0, 4°C) for 24 h changing the buffer solution more than 4 times. Complete removal of Cl^- from each sample solution was confirmed with silver nitrate test.

Isolation of thylakoid total lipids was done as shown in [13]. Depletion and reconstitution of PS II were taken as follows. The PS II particles were washed with buffer solution A, and then suspended in a solution B (1 M $CaCl_2$, 300 mM sorbitol, 10 mM NaCl and 40 mM Mes, pH 6.5, 4°C) at 1 mg/ml of chl [11] and stirred on ice for 30 min in darkness, followed by centrifugation at 30000 × g for 15 min at 4°C. The pellet was washed with buffer solution A (pellet/buffer, 1/10, v/v) containing 20 mM sodium cholate, recentrifuged at 30000 × g for 15 min and resuspended and homogenized in the same buffer solution to give a final [chl] of about 4 mg/ml. To this suspension, either one of the purified 17-, 23-, or 34-kDa proteins, or the lipids, or their combinations were added together with glycerol at a final concentration of 25 vol% and with buffer solution A containing sodium cholate at a final concentration of 15 mM. After incubation for 2 h at 4°C under gentle shaking, the mixed dispersion was diluted about 50 times with buffer solution A containing various amounts of NaCl to give a final [chl] of 8.3 μg/ml, and named RPS II. Control RPS II (CRPS II) was also prepared by the same method, with the addition of neither protein nor lipids to the medium of reconstitution.

Determination of manganese contents was done for the PS II particles and the differently prepared RPS II particles by means of atomic absorption spectrometry on a Perkin Elmer spectrometer (model 5000) equipped with a graphite furnace atomizer, as follows. Each sample was solubilized with Triton X-100 solution (2%) and 20 μl of the resulting solution was applied to the spectrophotometer, dried at 150°C for 10 s, ashed at 1000°C for 10 s and atomized at 2200°C for 5 s.

The measurement of O_2 evolution was carried out with PS II, RPS II and CRPS II samples under

various Cl^- concentrations by using a teflon-covered oxygen electrode (Bionics Instruments) at 25.0 ± 0.1°C. PBQ was used as an electron acceptor at 0.3 mM. Continuous illumination with saturating light (0.17 W/cm^2) between 600 and 800 nm was provided by a 1 kW tungsten-halogen lamp through a pair of color glass filters (Toshiba, R-60 and IRA-25S) and 10 cm water layer.

3. RESULTS AND DISCUSSION

In our previous paper, the effect of the thylakoid total lipids on the recovery of O_2 evolution in cholate-treated broken thylakoid membranes was reported [13]. A similar effect was observed in the $CaCl_2$-treated PS II particles. The recovery of O_2 evolution induced by simultaneous addition of the 17-, 23- and 34-kDa proteins without the total lipids was enhanced by further addition of the total lipids from approx. 40% of the original PS II to approx. 85% at the optimal concentration of the lipids (3 mg lipids/mg chl). Therefore, all of the reconstitutions were carried out with the total lipids at this concentration.

Fig. 1 shows the results of SDS–PAGE of the 3 proteins, PS II, $CaCl_2$-treated PS II (CRPS II) and RPS II prepared under different conditions. Each preparation of 17-, 23- and 34-kDa proteins gave a single band on the SDS–PAGE, indicating that they are free from contamination by other proteins. A comparison of the protein patterns of the PS II and CRPS II preparations revealed that the 17-, 23- and 34-kDa proteins were completely removed from the PS II particles by extraction with 1 M $CaCl_2$ as in [11]. The protein patterns of RPS II prepared with one of the 17-, 23- and 34-kDa proteins and with all of these proteins clearly showed that all of these proteins were rebound to the reconstituted membranes, distinct from the results shown in [12].

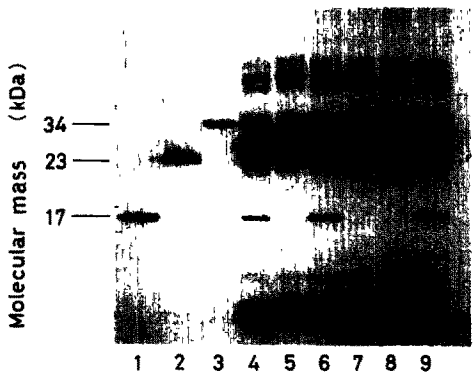

Fig. 1. SDS–PAGE of the proteins obtained from PS II particles upon various treatments. SDS–PAGE of 17-, 23- and 34-kDa protein preparations, PS II, CRPS II and RPS II formed under different conditions. All of PS II, CRPS II and RPS II were precipitated by centrifugation at 30000 × g and submitted to SDS–PAGE experiments: (1) 17-kDa protein; (2) 23-kDa protein; (3) 34-kDa protein; (4) PS II particles; (5) CRPS II particles: (6) RPS II particles (17-kDa protein and lipids); (7) RPS II particles (23-kDa protein and lipids); (8) RPS II particles (34-kDa protein and lipids); (9) RPS II particles (17-, 23-, 34-kDa proteins and lipids).

Fig. 2 shows the time course of O_2 evolution of PS II and RPS II. The high activity of the original PS II particles (a) was perfectly lost by 1 M $CaCl_2$ treatment (d), but it was restored in RPS II with 17-, 23- and 34-kDa proteins and total lipids at

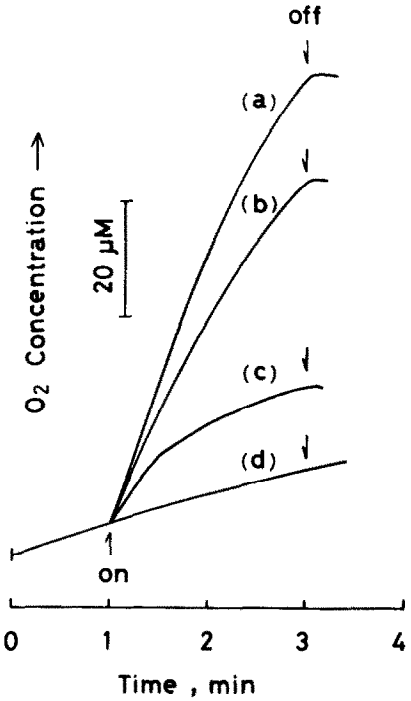

Fig. 2. Time course of O_2 evolution of PS II and RPS II particles: (a) PS II particles; (b) RPS II particles (17-, 23-, 34-kDa and lipids); (c) RPS II particles (34-kDa protein and lipids); (d) RPS II particles (lipids). The assays were carried out in the buffer solution A containing 20 mM NaCl.

about 85% of the original particles (b). The RPS II with the 34-kDa protein and lipids exhibited the O_2 evolution to a considerable extent during the initial 30 s as long as the assay medium contained Cl^- at a level of 20 mM, but after 30 s the rate of the O_2 evolution was much lower, as shown in (c). This abrupt decrease in the O_2 evolution rate after 30 s disappeared upon further addition of the 23-kDa protein indicating that the 23-kDa protein is also necessary to normal functioning of the water-splitting complex.

Fig.3 shows the effect of sodium chloride concentration, [NaCl], in the assay medium on the O_2 evolving activity of PS II and several RPS II prepared differently regarding the proteins. In the presence of 20 mM NaCl, both the RPS II prepared with the 23- and 34-kDa proteins, and the total lipids (△) and RPS II with the 17-, 23- and 34-kDa proteins and total lipids (○) exhibit high O_2 evolving activity (293 and 312 μmol O_2/mg chl per h, respectively). However, the O_2 evolving activity of the former gradually decreased with decreasing [NaCl] and sharply dropped toward zero when the concentration was less than 2 mM. While the activity of the latter remained constant, independently of [NaCl], until 2 mM, and began to decrease with decreasing [NaCl] at less than 2 mM, it remained at 178 μmol O_2/mg chl per h, even when no NaCl was added to the assay medium. If the activity was calculated from the initial rate, RPS II prepared with the 34-kDa protein and the total lipids exhibited fairly large O_2 evolving activity (202 μmol O_2/mg chl per h) at 20 mM NaCl but it declined with decreasing [NaCl] and reached zero at 2 mM NaCl. RPS II prepared with the 17- and 34-kDa proteins and the total lipids showed essentially the same behavior as the RPS II with the 34-kDa protein and the total lipids (□). RPS II prepared with the 17-kDa protein or the 23-kDa protein and the total lipids did not show any O_2 evolving activity over the whole range of [NaCl] examined. These results led to the conclusion that the 34-kDa protein is essential for the recovery of O_2 evolution in the $CaCl_2$-treated PS II and the 23-kDa protein is also required for normal functioning of the water-splitting complex even when NaCl exists at a level of 20 mM in the assay medium. Furthermore, when [NaCl] drops, in particular below 2 mM, besides the 34- and 23-kDa proteins, the 17-kDa protein is required for the O_2 evolution. These facts are able to explain the discrepancies among the early works regarding the requirement for the 17-, 23- and 34-kDa proteins, as pointed out in [13].

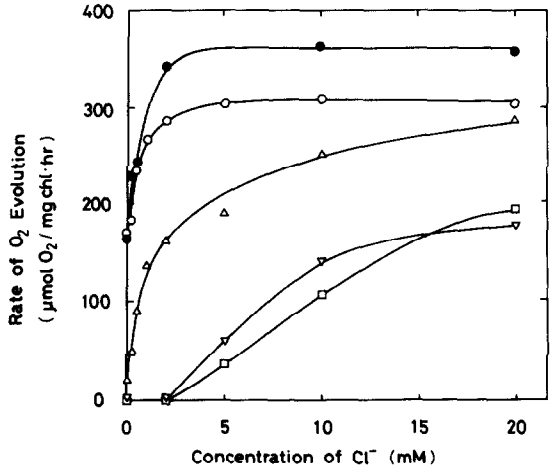

Fig.3. Effect of NaCl concentration on the O_2 evolving activity of PS II and RPS II particles prepared under various conditions: (●) PS II particles (the particles were washed with buffer solution A prior to the assay); (○) RPS II particles (17-, 23-, 34-kDa proteins and lipids), 17-kDa protein/23-kDa protein/34-kDa protein/lipids/chl ratio, 0.45/0.60/0.60/3.0/1.0 (by wt); (△) RPS II (23-, 34-kDa proteins and lipids), 23-kDa protein/34-kDa protein/lipids/chl ratio, 0.6/0.6/3.0/1.0 (by wt); (▽) RPS II (17-, 34-kDa proteins and lipids), 17-kDa protein/34-kDa protein/lipids/chl ratio, 0.45/0.60/3.0/1.0 (by wt); (□) RPS II (34-kDa protein and lipids), 34-kDa protein/lipids/chl ratio, 0.60/3.0/1.0 (by wt). The assays were carried out in buffer solution A containing various amounts of sodium chloride.

The amount of manganese retained in the $CaCl_2$-treated PS II particles which totally lost three proteins was about 80% of that of the original PS II on average. Therefore, the difference in the O_2 evolving activity between the original PS II and the RPS II prepared with the 17-, 23- and 34-kDa proteins and the total lipids is considered to be due to a slight removal of manganese from the PS II during the $CaCl_2$-treatment. In other words, for the 17-, 23- and 34-kDa proteins, the disintegration and reconstitution are performed perfectly by the present method which may provide an advantage in studying the molecular events in the water-splitting process.

So we applied the present method to examine the relation between the amount of manganese retained in the water-splitting complex and the O_2 evolving activity. To remove manganese from PS II particles in various degrees, PS II particles were incubated in OG solution with various concentrations (0.2 M sucrose, 20 mM Mops, 0–40 mM OG, pH 7.0) at 0°C for 1 h under gentle stirring, followed by centrifugation at 145 000 × g for 30 min. By using this pellet, the reconstitution was carried out with the 17-, 23- and 34-kDa proteins and the total lipids as before. In fig.4, the O_2 evolving activities of the resulting RPS II and RPS II(OG) were plotted against the amounts of manganese retained in the corresponding RPS II. The plots fall on a straight line through a point corresponding to the original PS II (□) and the O_2 evolving activity was totally lost when the manganese contents were reduced to a value of 3.4 Mn/400 chl (mol ratio). These results suggest that the existence of approx. 10 Mn/400 chl is required for the O_2 evolution. Two-thirds of the manganese is solubilized relatively easily by OG-treatment concomitant with the loss of the O_2 evolving activity, but the remaining 1/3 will not be liberated from the PS II by treatment with 40 mM OG for 1 h, although it cannot activate the O_2 evolution by itself. Consequently, it may be supposed that there are at least two different types of manganese binding protein in the water-splitting complex.

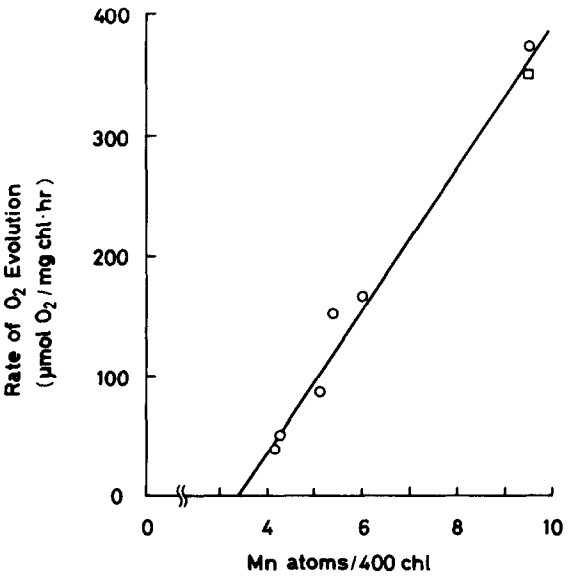

Fig.4. Relationship between the amount of Mn retained in the membranes and the O_2 evolving activity. (○) RPS II(OG) (17-, 23-, 34-kDa proteins and lipids), 17-kDa protein/23-kDa protein/34-kDa protein/lipids/chl ratio, 0.45/0.60/0.60/3.0/1.0 (by wt); (□) PS II. The reconstitutions were carried out by the same method as for the $CaCl_2$-treated particles.

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